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"LEUCOCYTOSIS IN PLAGUE AND OTHER INFECTIONS" AND "A METHOD EMPLOYED IN STUDYING THE INTERACTION OF BACILLI AND ANIMAL FLUIDS 'IN VITRO'."

By Drs. R. Row, M.D., B.Sc. (London) and
N. F. Surveyor, M.D., M.A.

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"LIFE-HISTORY OF THE MALARIAL PARASITE."

By Dr. Ronald Ross, F.R.S.

During the last few years I have had considerable opportunities of studying the ratio between the sexes of the parasite is concerned, and although a great many observations have at present been made, they are not always easy to interpret.

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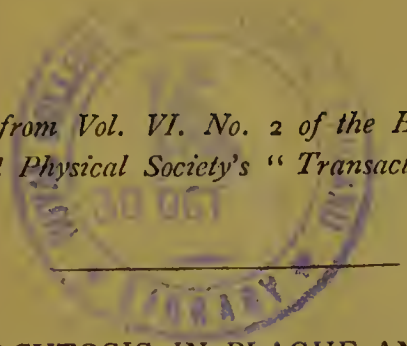

"LEUCOCYTOSIS IN PLAGUE AND OTHER INFECTIONS."

By Drs. R. Row, M.D., B.Sc., and N. F. Surveyor, M.D., M.A.

During recent years Leucocytosis in disease has received considerable attention from various workers, especially as far as the ratio between the Polymorphic and Mononuclear leucocytes is concerned. Actual increase or diminution of leucocytes, although a very important phenomenon, has not been studied by many observers, simply because the methods that are available at present are still defective, and the results obtained are thus not always uniform.

In the work which we lay before you, we have also confined ourselves to the determination of the ratio between the Polymorphic and Mononuclear leucocytes in diseases that are commonly classed as septic or infective.

However, we have to make a few remarks on what we venture to term "Leucocytic Motility" in the blood of healthy and diseased persons. In the beginning of 1896 one of us (S.) started some observations on the fresh blood of malarial cases with a view to study the malarial parasites and see if these would undergo any further development on the microscope slide if the blood was kept sterile for several hours or even days. Thus it was found quite easy to keep a slide sterile for about a week, and, owing to the warm climate in which the work was done, it became soon evident that the leucocytes retained their amœboid character for several hours in healthy blood. The method of preparing these slides is in no way peculiar, except that perfect cleanliness is observed and the margin of the cover-glass is sealed up with pure vaseline. The drop of blood must be about twice the

size one ordinarily takes for studying the malarial parasite, or else the leucocytes get compressed between the slide and the cover-glass and their amœboid movements become very sluggish. The leucocytes, as a rule, in healthy persons have been found to retain their motility for about twenty-four to forty-eight hours, while in one case the longest period noted was eighty hours. The ordinary finely granular Polymorphic forms generally die at the end of twenty-four hours, while the Eosinophiles are the most resistant ones.

This motility varies considerably in disease. Thus observations in septic conditions, which primarily affect the blood, have shown that the motility is generally abolished within three to six hours. On the other hand, in septic infection of the peritoneum, which, as it were, kills the patient too rapidly, no time is allowed for the leucocytes to be much affected, and in such cases the leucocytic motility, although less than normal, is not so rapidly destroyed ; in fact in some cases the leucocytes have been found to be slightly motile for about three to four hours after death of the patient, provided the slide be prepared soon after or before death.

On the other hand, in specific fevers like Enteric, Relapsing, Plague, etc., the motility varies more with the duration and severity of the case and also the vitality of the person than only with the rise of temperature. Thus, although the temperature be the same in two cases, one will show good amæboid motility, while in the other case motility may be quite abolished from the beginning or may be lost within a few hours. Thus in two cases of Enteric fever, although the temperature was 99·8° F. on a particular day, the leucocytic motility continued for twenty-four hours in one case, while in the other it was scarcely seen. Both cases were of about the same age. The former case had just passed through the whole of the febrile course, and the latter was in the middle of the attack. On the other hand, very good leucocytic motility has been observed in Plague and Relapsing fever at the commencement of the disease, even with very high fever. Of course in such cases they

do not remain motile for more than six to twelve hours; yet, soon after preparing the slide, the motility, if not quicker than in normal persons, is at least equal to it. The quickening of the motility may probably be due to the irritant action of the toxins on the previously healthy leucocytes. If the disease progresses unfavourably the leucocytic motility gets abolished, and, on the other hand, it becomes re-established as recovery takes place.

Besides this alteration of motility certain other phenomena have been observed to take place in the Polymorphic, large Mononuclear and Eosinophile cells.

These consist in the formation of vacuoles, sooner or later according to the gravity of the infection. Thus in cases of advanced phthisis the leucocytes are more or less free from granules and are full of vacuoles, even within a few minutes of preparing the slide. In such cases the leucocytes show very feeble motility, and the vacuoles are large in size.

On the other hand, in some cases of Plague and Relapsing fever, during the early part of the disease, the vacuolation is quite peculiar; in fact it is not quite easy to be certain whether to call these round spaces real vacuoles, or to look upon them as some peculiar hyaline degeneration of the protoplasm of the cell. By transmitted light they appear more refractile than the vacuoles seen in phthisis and have a greenish pink border. When the slide is examined soon after its preparation, we find these round globules rolled about in the body of the leucocytes, which are almost twice the size of normal cells and show good amæboid motility.

In some favourable cases we may find the cell undergoing dissolution, and in such cases the real nature of these globules becomes evident, *i.e.*, they are vacuoles, because at this stage these show appearances similar to what one finds in vacuolated dead leucocytes.

On the other hand, in some cases these leucocytes shrink while they are being watched, and in such cases the vacuoles seem to disappear. The protoplasm, which is already very granular, becomes more so, and for some hours short spiny pseudopodia are only thrown out.

That what we have described must be vacuoles, is also evident from the fact that the shrinkage of the leucocytes in process of drying and fixing obliterates these in most cases, this being true of Phthisis, Plague, Relapsing Fever, Enteric Fever, and any other debilitated condition in which these vacuoles are found from the commencement. In healthy blood many of the leucocytes become vacuolated after about twelve to twenty-four hours, and in such a case one can wipe off the vaseline, slide off the cover-glass, and make a permanent specimen showing large vacuoles.

When these plague vacuoles were first seen by one of us (Dr. R.), it was thought that, perhaps, it may be a specific effect of the plague toxins on the leucocytes, however its occurrence in a case of Relapsing Fever, and its non-occurrence in some cases of plague renders any such view untenable. However, one good result of the surmise has been the working out of a new method for the study of the interaction of animal fluids and bacilli.

It has been stated above that in some cases the vacuoles disappear owing to the shrinkage of the leucocytes, which become more granular and spiny pseudopodia are formed. This latter phenomenon we have obtained by directly adding a drop of very dilute cultures of the plague bacilli in normal saline to fresh healthy blood. However, up to now we have not succeeded in obtaining the vacuole formation similar to what has been described above. In healthy blood one often finds vacuoles after about eight to twelve hours in some leucocytes, but generally they are very few in number, about one or two vacuoles being seen in a single leucocyte, and even these are

small in size. In diseased blood, on the other hand, one may find a leucocyte full of them, and in some cases almost all the leucocytes are full within a short period of preparation of the slide.

Besides vacuole formation, we have observed a peculiar dancing movement of the granules of the leucocytes, especially the Eosinophiles, whose significance we have not yet been able to decide upon.

At first sight it appears to be a sort of brownian movement of these granules, due to the altered condition in which the leucocytes are placed as regards the surrounding plasma, this probably producing osmotic currents in the body of the leucocytes. On this hypothesis it was decided to try the effect of a drop of distilled water on a slide prepared for the study of the leucocytic motility. The result is quite identical as far as the movement of the granules is concerned. The leucocyte at once becomes rounded and quiescent, and these granules begin to show active brownian (?) movement. The leucocyte, however, within a few minutes bursts and sets free these granules. In fact, in some text-books of Physiology this experiment has been described as showing non-vital or brownian movement of particles.

However, there are other facts which make us hesitate before accepting such a simple explanation of this phenomenon. Thus, in some cases, as the leucocytes are dying, one sees these granules moving slowly from one place to another in the body of the cell, which is perfectly quiescent for a short time ; then these recommence to oscillate rapidly. Again, they stop all movement for a short time, and the vibratile movement commences once more. This may continue for some hours. About four hours is the longest period for which this process has been watched, the leucocyte itself showing slow amœboid movement at the same time, thus proving that the cell is not dead. This phenomenon may last for a very few minutes in some cells. The finely granular cells also show the same movement, just

like the Eosinophile cells. This movement seems to come on in some septic cases within a very short time of preparing the specimen. In healthy blood it comes on sometimes after about twenty-four hours.

Again, one reason why this phenomenon cannot be a merely mechanical one, dependent upon osmotic action, is the fact that all the leucocytes do not show it at the same time in a particular specimen. A few cells will show it, while others will show good active amœboid movement and no vibration of the granules. We have not been able to see this phenomenon in every specimen. However, we venture to think that it is a universal one, and that one must, as it were, catch the leucocyte at the right moment. Sometimes the granules vibrate for a very few minutes, and so one does not get an opportunity of watching it. Were it possible to watch a specimen continuously, we think it quite likely that every specimen would show it. As regards the explanation of this phenomenon, we venture to suggest that it is like the fibrillar contraction of a dying muscle. In fact, we may describe it as the twitching of the dying protoplasm, and that it seems to come on sooner and continue for a short time only in cases where the leucocytes die early as in advanced phthisis.

These observations as regards leucocytic motility, vacuole formation, and the peculiar granular movement of the leucocytes seem to make it possible that in certain diseases we may be able to give a favourable or unfavourable prognosis, or to decide whether or not the general condition of the patient is improving under a certain course of treatment. In fact, it can be used as a gauge of the vitality of a patient.

As regards the ratio of the Polymorphic to Mononuclear leucocytes in various diseases, we have completed our observations in plague cases, and these we are able to lay before you to-day. The first most prominent feature noticed is the almost complete abolition of Eosinophile cells in the majority of cases.

The next thing to draw one's attention is the increase of the large hyaline Mononuclears as compared with the lymphoctyes.

As regards the ratio between the Polymorphic and Mononuclear leucocytes as compared with the lymphocytes, we have found it to vary considerably, and we think that the series of observations must be considerably increased before it can be possible for us to say whether the increase of the Polymorphic cells is a favourable or unfavourable sign, showing greater (or less capacity for resistance) than in cases where the reverse is the condition.

Cases Nos. 1, 2, 3 and 8 seem to show, on the other hand, that considerable increase of lymphocytes, even a reversal of the ratio, is not unfavourable to recovery, all these cases having recovered.

One thing, however, seems to be certain, that as death approaches the ratio tends to approach the normal, and that in very grave cases, as in case No. 11, no reaction altering the ratio seems to be produced.

Before concluding our paper we have to make a few remarks about the lymphocytes.

These are generally regarded by physiologists as non-amœboid cells. Coles, in his work on "Diseases of the Blood," definitely states that they are non-amœboid. While studying the leucocytic motility of the white cells, one of us (S.) has observed that the lymphocytes, which are generally not amœboid when the blood is fresh, become distinctly amœboid after about twelve to twenty-four hours in healthy blood, while in some septic cases, or even in normal blood, a few have been found motile even in fresh blood.

Again, when counting the leucocytes in stained preparations, one often comes across a stained lymphocyte fixed in an amœboid form. This was at first thought to be due not to active amœboid state of the lymphocyte at the time of being fixed,

but to stretching of the protoplasm in smearing the blood on the slide. However, one can easily disprove this supposition by the fact that in some fields of the specimen these stretched lymphocytes are found side by side with round ones showing no alteration in form, and again the direction in which the former are stretched is not the same.

Major Herbert, in his paper on "The young Plasma Cell or Lymphocyte in Chronic Inflammation," states that "lymphocytes are commonly classed as non-amœboid. In chronic trachoma and follicular conjunctivitis, however, many of these young cells, distributed through the membrane, are stimulated into active movement. The nuclei stain darkly and uniformly, while the thin coverings of protoplasm are no longer visible with the basic dyes. The softened, pliable cells stretch out along the tissue spaces, often into long threads, accommodating themselves to the passages by which they travel away." Thus in stained sections he has found them variously shaped.

The reason why in fresh blood these have not been detected to show amœboid movement, is probably due to the fact that they are either very few or exist in very young state, while they become motile as they grow older and undergo further development *in vitro* as they probably do *in vivo* on their way to become large hyaline Mononuclear leucocytes, as has been surmised by Professor Sherrington.

We have not been able to finish the counting of leucocytes in other infections yet, so we have had to divide our paper into two parts : the second part will be published as soon as possible. Up to now we have examined the blood of about 150 cases for the study of leucocytic motility, vacuolation, etc., in fresh specimens.

Table showing Leucocytosis in Plague Cases.

No.	Polymorphic Leucocytes.	Eosinophile Cells.	Hyaline Leucocytes.	Lymphocytes.	Day of Illness.	Remarks.
1*a*	45·1	0·2	11·8	42·9	3rd	T. 102·8. Age 10 years. Condition good. Leucocytic motility very good. Large vacuoles. Bubo in left groin.
1*b*	38·7	0·6	8·7	52·0	7th	T. 98. Blood platelets in large numbers. Vacuoles few.
1*c*	27·5	1·2	3·6	67·6	14th	Bubo incised. Convalescent. Vacuoles very few.
2*a*	76·8	...	5·6	17·4	2nd	Age 12 years. Delirium present. Condition good. Leucocytic motility sluggish. Vacuoles considerable. Bubo in left axilla.
2*b*	59·8	1·6	5·6	31·5	11th	Condition good. Convalescent. Only a few leucocytes are vacuolated. Some show vibrating granules.
3*a*	58·2	...	3·5	31·1	4th	Condition good, Leucocytes full of vacuoles and slow in motility. Bacilli present in blood. Bubo in left groin.
3*b*	57·4	1·75	5·3	35·5	11th	Bubo incised. Condition convalescent. Vacuoles present. Vibrating granules in leucocytes.
4	83·3	...	5·9	10·8	4th	T. 102. Leucocytes full of vacuoles. Bubo in groin. Hyalines large in size, like a fatal case of Spirillum fever. Died on 5th day.
5*a*	59·2	...	8·2	32·6	3rd	
5*b*	84·5	...	1·5	14·0	5th	
5*c*	64·6	0·5	4·7	30·5	7th	Died 3 hours after the sample of blood was taken.
6*a*	55·2	...	2·9	42·7	3rd	Bubo in right groin.
6*b*	56·3	...	1·3	42·4	4th	
6*c*	58·6	...	2·3	39·1	5th	
6*d*	71·8	0·5	1·35	28·3	6th	
6*e*	40·3	...	1·8	57·9	8th	Died on 8th day.
7*a*	57·0	...	3·0	40·0	4th	Bubo in right inguinal region.
7*b*	68·6	...	5·6	26·0	5th	Died on 5th day.
8*a*	42·6	0·2	6·2	51·2	...	Right axillary bubo.
8*b*	46·6	...	0·6	53·0	...	Convalescent.
9*a*	58·0	0·7	5·4	36·0	4th	Right axillary bubo.
9*b*	55·5	2·7	4·7	37·0	5th	Died on 5th day.
10*a*	82·0	...	6·0	12·0	4th	3rd day of bubo.
10*b*	83·1	...	6·2	10·7	5th	Died on 5th day. No vacuoles present. Leucocytes slow, contracted, short spiny pseudopodia seen. Motile for 12 hours.
11	66·4	0·1	5·1	29·4	1st	Died on 2nd day. Leucocytic motility very feeble ; lasted for 5 hours. No typical vacuoles. T. 105·6. P. 138, R. 32. Bubo appeared next day in groin.
12*a*	86·8	...	2·2	11·0	2nd	Leucocytic motility lasted for 4 hours. No typical vacuoles at first : however, all became vacuolated after 4 hours. T. 103·6. P. 120. R. 28.
12	83·0	...	5·2	12·8	4th	Leucocytic motility very feeble ; present for 10 hours. Most became vacuolated after a short [time.
13	85·7	...	3·1	11·2	4th	Plague Pneumonia. T. 105·8. P. 140. R. 35. Leucocytic motility fairly good. Not much vacuolation present. Motile for about 8 hours.

"A METHOD EMPLOYED IN STUDYING THE INTERACTION OF BACILLI AND ANIMAL FLUIDS 'IN VITRO.'"

By Drs. R. Row, M.D., B.Sc., and N. F. Surveyor, M.D., M.A.

The serum of animals, either in coagulated or liquid state, has been used for the cultivation of bacteria. Again, this fluid has been added in various forms to other nutrient media with a view to increase their nutritive value. However, the classical observations of Widal, on the Agglutination of Typhoid Bacilli by the Serum of Typhoid cases, have opened up a new line of research.

Recently Professor Wright (1) and Dr. Leishman (2) of Netley have published some observations describing new methods of studying the effect of bacilli in blood *in vitro*. Professor Wright uses dilute cultures of bacilli in gelatine. These are mixed with the sera of *persons immunised* against typhoid. This mixture is made in capillary pipettes, which show on incubation the formation of small colonies, and these can be easily counted under the microscope. These vary in number according to the greater or less immunity produced in the individual whose blood has been used. Dr. Leishman (2) has recently published a paper, in which he shows how easy it is to obtain phagocytosis in human blood *in vitro* by simply mixing fresh blood with a small amount of a culture of any micro-organism. With this short review of the work already done by other workers before us, we will describe the method adopted by us for studying the interaction of bacilli and animal fluids, chiefly blood.

In June last, as we have already stated in our paper on "Leucocytosis in Plague and other Infections," it occurred to one of us (S.) that as blood can be kept sterile for several days on slides, we might mix a very dilute culture, either live or dead, of plague bacilli with live blood, and try to obtain leucocytic vacuolation in healthy blood, a phenomenon found by Dr. Row in the blood of plague cases. The first experiments were made with bouillon cultures

of the plague bacillus. However, the result was not satisfactory. Probably the cultures were very active, as the leucocytes were killed in a very short time. The longest time they lived was about six hours. The most marked effect noticed by us was the contracted state of the protoplasm and formation of short spiny pseudopodia. In order to study the effect of less concentrated cultures, we have made very dilute emulsions of young agar cultures in normal saline. A very minute particle of the culture is taken up on a platinum needle, and this is mixed with about 10 cc. of sterilised normal saline. A very small drop of this is taken up with a capillary pipette or on a platinum loop, and this is well mixed with fresh blood or serum which has been standing for various periods. From this mixture a sterile slide is made in the usual manner, the edges of the cover-glass being fixed with vaseline. At first not a trace of bacilli is found. However, after six to twelve hours in favourable sera these grow very rapidly; in fact, after about twenty-four hours the specimen becomes full of strepto-bacilli. The leucocytes, which appear to be slightly stimulated at first, become very sluggish. In fact, they become contracted and form short spiny pseudopodia. Phagocytosis, according to Dr. Leishman's method, was looked for, however, very few leucocytes were found to have taken up the bacilli. This may be due to the great toxicity of the cultures. With Bacillus Coli we have obtained good phagocytosis under similar conditions. The strepto-bacilli seem to increase in number very rapidly. However, after about forty-eight hours, very few bacilli could be seen, this being due probably to the exhaustion of the nutrient medium, leading to disintegration or plasmolysis of the bacilli. With the serum of different individuals we found that, under similar conditions, the formation of strepto-bacilli varied considerably, thus showing that the blood of different persons varies in nutrient and immunising qualities.

This method, however, has some serious objections—

(*a*) It is non-demonstrable.

(*b*) It only allows the growth of bacilli in anœrobic condition.

(*c*) It is dangerous when pathoginic microbes are used.

(*d*) The slides may become contaminated.

However, these defects have been overcome by one of us (R.) using hanging drop slides. By this method it has been found quite easy to obtain preparations which can be examined under the microscope for any length of time. Again, the growth can be demonstrated in stained preparation. For this we have only to slip off the cover-glass, allow the drop to dry, wash off the vaseline and stain.

In this method there is one defect, which however is a matter of detail, *viz.*, the use of vaseline for fixing the cover-glass to the hollow slide. Removing the vaseline is not at all an easy process, nor is the handling of thin cover-glasses at time of preparing the slide free from risk.

To obviate these objections a modification has been made in the process (S.), although the principle remains the same. Slides or cover-glasses can be sterilised in suitable broad test-tubes, and drops of infected blood can be put on these. In the case of slides, several drops can be put on a slide, so as to have control preparations under similar circumstances. The test-tubes then can be put horizontally in a suitable stand.

By this method we do away with the necessity of using vaseline. However, the tubes must be kept in a moist atmosphere, or else the drops dry up in a very short time. For this purpose a pledget of moist cotton-wool can be put either at the bottom of the tube at time of sterilising the tubes in the autoclave, or, after making the drop culture, a sterilised plug moistened with sterile water can be introduced into the test-tube.

A further precaution to prevent evaporation is to place the tubes in such a position, so that the drops remain on the under-surface of the slide or cover-glass.

At the time of staining the preparation, one has only to pull out the slide or cover-glass with a pair of forceps, dry, fix and manipulate in the usual manner. However, when one wants to study a fresh specimen under the microscope, either the hanging drop method must be adopted, or else the cover-glass must be removed from the test-tube and placed over a hollow slide, using a little vaseline to fix one of the corners. A square cover-glass is most convenient for this purpose.

By adopting these steps we have been able to obviate most of the difficulties we have encountered, though we cannot say that the method is yet perfect.

In conclusion, we may state that we were not aware of the work of Professor Wright and Dr. Leishman till we had nearly worked out our method.

(1) On a Method of Measuring the Bactericidal Power of the Blood for Clinical and Experimental Uses. By Professor A. E. Wright, Professor of Pathology, ArmyMedical School, Netley.—*Lancet*, p. 1556, Vol. II., 1900.

(2) Note on a Method of Quantitatively Estimating the Phagocytic Power of the Leucocytes of the Blood. By Major W. B. Leishman.—*British Medical Journal*, p. 73, Vol. I., 1902.

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